IOP-Dependent Retinal Ganglion Cell Dysfunction in Glaucomatous DBA/2J Mice

Mahesh Nagaraju, Maher Saleh, and Vittorio Porciatti

Purpose. To characterize the effect of postural IOP elevation and pharmacological IOP lowering on retinal ganglion cell (RGC) function in the DBA/2J mouse model of glaucoma.

Methods. Four groups of DBA/2J mice (3 months old, $n = 7$; 5 months old, $n = 7$; 10 months old, $n = 7$; and 11 months old, $n = 8$) were anesthetized by intraperitoneal injection (0.6 mL/kg) of a mixture of ketamine (42.8 mg/mL), xylazine (8.5 mg/mL), and acepromazine (1.4 mg/mL). IOP and pattern electroretinogram (PERG) were sequentially measured with mice at 0° (horizontal), 60° head-down, and again at 0°. IOP and PERG were also measured before and after intraperitoneal mannitol 25% (2.5 g/kg) administration with mice in a horizontal position.

Results. The head-down position induced reversible IOP elevations of 32% to 38% in all age groups ($P < 0.01$), and age-dependent reductions of PERG amplitude (3 months: +3%; 5 months: −47%, $P < 0.01$; and 10 months: −65%, $P < 0.01$). Administration of mannitol to 11-month-old mice resulted in a reduction in IOP of approximately 38% ($P < 0.01$) and a PERG amplitude improvement of approximately 83% ($P < 0.001$). IOP and PERG amplitude changes were inversely correlated (10 months head-down $r^2 = 0.58$, $P < 0.001$; 10-month-old mannitol $r^2 = 0.41$, $P < 0.001$). For all conditions, the light-adapted flash ERG was unaltered.

Conclusions. In the DBA/2J mouse, RGC susceptibility to artificial IOP elevation increases with age. Abnormal RGC function in older mice may be improved with IOP lowering. Evaluation of PERG changes in response to artificial IOP modulation may represent a powerful tool to assess noninvasively RGCs' susceptibility to IOP insult in genetically distinct mouse models of glaucoma. (*Invest Ophthalmol Vis Sci.* 2007;48:4573–4579) DOI:10.1167/iovs.07-0582

Mouse models with genetic alterations relevant to glaucoma are receiving increasing attention in an effort to gain better understanding of the complex nature of the disease and to design specific treatments to prevent death of retinal ganglion cells (RGCs) and their axons.[1–4]

The DBA/2J mouse is a well-established model of spontaneous glaucoma. Recessive mutations in two genes, *Gpnmb* and *Tyrp1*, cause iris atrophy and pigment dispersion.[5] The iris disease is apparent at 6 months and progresses with age, resulting in elevated intraocular pressure (IOP), loss of RGCs and optic nerve axons, and optic disc excavation.[6] Young (2–3-month-old) DBA/2J mice have normal IOP and normal histologic appearance of RGCs and optic nerves. By 8 to 9 months of age, the RGCs show signs of apoptotic death.[7,8]Axonal damage in the optic nerves is apparent in approximately 50% of eyes by 10 to 11 months of age and in approximately 90% of eyes by 18 months.[6]

Surviving RGCs may not be functional. Functional events associated with IOP elevation and RGC progressive degeneration in DBA/2J mice should be elucidated. RGC function in mice can be evaluated by means of the pattern electroretinogram (PERG).[9–12] The noninvasive nature of the PERG allows serial recordings as a function of changing conditions (e.g., age, IOP levels). Using PERG, we have been able to characterize the natural history of RGC dysfunction and its association with IOP in a 12-month longitudinal study of BDA/2J mice (Saleh M et al. *IOVS* 2007;48:ARVO E-Abstract 210). On average, the IOP increased from 14 to 18 mm Hg between 2 and 6 months and then more steeply, leveling off by 11 months at approximately 28 mm Hg. After 3 months, the PERG amplitude decreased progressively with age to approach the noise level at approximately 10 to 11 months. The time-course of IOP elevation and PERG amplitude reduction were closely associated. Histologic analysis of eyes with abolished PERG showed that the retinal nerve fiber layer (RNFL) had lost approximately 40% of its normal thickness and the cone-flash ERG did not significantly change (Saleh M et al. *IOVS* 2007;48:ARVO E-Abstract 210). Taken together, these results indicate that DBA/2J mice have progressive functional damage in the inner retina (abnormal PERG) but not in the outer retina (normal flash-ERG) that seems to precede anatomic damage of the optic nerve (relatively spared RNFL).

Recently, Aihara et al.[13] reported that in NIH white Swiss mice the head-down position induces substantial elevation of IOP associated with elevation of the episcleral venous pressure (EVP). The head-down position induces very similar IOP and EVP changes in humans (e.g., Ref. 14) In the present study, we tested the hypothesis that IOP elevation in the head-down position causes reduction in PERG amplitude in susceptible eyes of older DBA/2J mice. We also tested the hypothesis that impaired RGC function in older DBA/2J mice may be improved by pharmacologically reducing IOP with mannitol.[15] Preliminary results of this study have been presented earlier in abstract form (Nagaraju M et al. *IOVS* 2007;48:ARVO E-Abstract 212).[16]

Methods

Animals and Husbandry

A total of 29 DBA/2J mice of different ages—3 months, $n = 7$ (7 eyes); 5 months, $n = 7$ (7 eyes); 10 months, $n = 7$ (7 eyes); and 11 months, $n = 8$, (16 eyes)— obtained from Jackson Laboratories (Bar Harbor, ME) were tested. The mice were maintained in a cyclic light environment (12-hour 50 lux, 12-hour dark) and fed ad libitum. For both IOP and PERG measurements, the mice were weighed and anesthetized with intraperitoneal injection (0.5–0.7 mL/kg) of a mixture of ket-

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amine, 42.8 mg/mL; xylazine, 8.6 mg/mL; and acepromazine, 1.4 mg/mL. All procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Animal Care and Use Committee of the University of Miami.

IOP Measurement

IOP was measured with an induction–impact tonometer (Tonolab Colonial Medical Supply, Franconia, NH).[17] The tonometer was fixed in a vertical position to a support stand by means of clamps. The anesthetized mice were placed on an adjustable stand and the tail restrained with adhesive tape. A magnifier lamp was used to align the probe tip with the optical axis of the eye at 1- to 2-mm distance. Five consecutive IOP readings were averaged. The impact of the Tonolab probe on the cornea is minimal, so that local corneal anesthesia is not necessary.[18] In a previous longitudinal study over 1 year (Saleh M et al., *IOVS* 2007;48:ARVO E-Abstract 210), we did not find obvious corneal damage in biomicroscopic examinations. IOP readings obtained with Tonolab have been shown to be accurate and reproducible in different mouse strains including DBA/2J (Saleh M et al., *IOVS* 2007;48:ARVO E-Abstract 210).[18]

PERG Recording

A detailed description of the PERG technique is available elsewhere.[11,12] In brief, the anesthetized mice were gently restrained by using a bite bar and a nose holder that allowed unobstructed vision[11,19] and kept at a constant body temperature of 37.0°C with a feedback-controlled heating pad. The body of the mouse could be oriented horizontally as well as 60° head-down on an adjustable platform by holding the tip of the tail with adhesive tape. The eyes of anesthetized mice were typically wide open and in a stable position, with undilated pupils pointing laterally and upward. The ERG electrode (0.25-mm diameter silver wire configured to a semicircular loop of 2-mm radius) was placed on the corneal surface by means of a micromanipulator and positioned in such a way as to encircle the undilated pupil without limiting the field of view. Reference and ground electrodes were stainless-steel needles inserted under the skin of the scalp and tail, respectively. A small drop of balanced saline maintained the cornea and lens in excellent condition for the duration of recording. A visual stimulus of contrast-reversing bars (field area, 50° × 58°; mean luminance, 50 cd/m^2; spatial frequency, 0.05 cycles/deg; contrast, 98%; and temporal frequency, 1 Hz) was aligned with the projection of the undilated pupil at 20 cm distance. Eyes were not refracted for the viewing distance, because the mouse eye has a large depth of focus due to the pinhole pupil.[20,21] Retinal signals were amplified (10,000-fold) and band-pass filtered (1–30 Hz). Three consecutive responses to 600 contrast reversals each were recorded. The responses were superimposed to check for consistency and then averaged. The PERG is a light-adapted response. To have a corresponding index of outer retinal function, a light-adapted ERG (FERG) was also recorded with undilated pupils in response to strobe flashes of 20 cd/m^2/s superimposed on a steady background light of 12 cd/m^2 and presented within a Ganzfeld bowl. Under these conditions, rod activity is largely suppressed while cone activity is minimally suppressed.[22] Averaged PERGs and FERGs were automatically analyzed to evaluate the major positive and negative waves (Fig. 1). For both PERG and FERG, peak-to-trough response amplitudes were automatically evaluated on the major positive–negative complex, and the latency was the time to peak of the major positive deflection. Separate analysis of positive and negative components of responses was not systematically performed because unambiguous recognition of these components was not possible in older mice with reduced PERG signal.

Pupil Size

Anesthesia may cause pupil dilation,[23] and older DBA/2J mice may have slight pupil dilation or deformation due to the pathologic iris condition.[5] The size of the pupil was continuously checked with a magnifier lamp during sequential IOP measurements. Pupil size was also checked with a microscope (25×) at the beginning and at the end of the PERG recording together with assessment of correct positioning of ERG electrodes. A four-point grading system was used to describe the approximate pupil size: small, slightly dilated, moderately dilated, and dilated. All mice had small or slightly dilated baseline pupils. The pupil size did not appreciably change after either head-down posture or mannitol administration.

Experiment 1: IOP Elevation

After two baseline IOP measurements performed with the animal resting in a horizontal position, the adjustable platform was tilted in such a way as to put mice in a head-down (60°) position for 30 minutes and then repositioned horizontally for at least 20 minutes (recovery). Sequential IOP measurements (1–5 minutes apart) were obtained over the whole tilt–recovery period. The adjustable platform was then inserted in the stereotaxic holder for PERG recording. Since recording time for PERG/FERG recording was approximately 15 minutes, only three sequential responses could be obtained during the observation period: baseline, 15 to 30 minutes after placement in the head-down position, and 15 to 30 minutes after resuming the horizontal position.

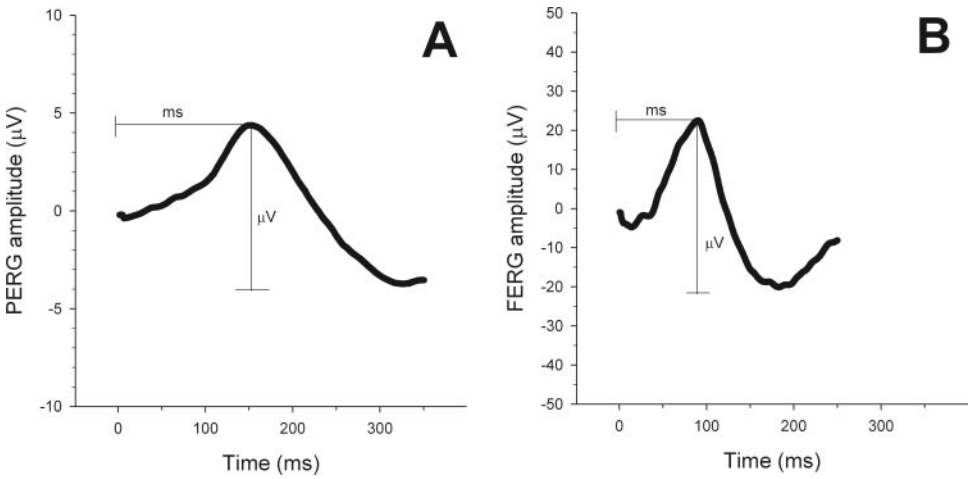

Figure 1. Examples of PERG (**A**) and FERG (**B**) recorded in DBA/2J mice aged 3 months. For both PERG and FERG, the amplitude was measured from the positive peak to the negative trough. The latency was evaluated from the stimulus onset (contrast reversal for the PERG, strobe flash for the FERG) to the peak of the positive wave. Note that the PERG had smaller amplitude and longer latency compared to the FERG.

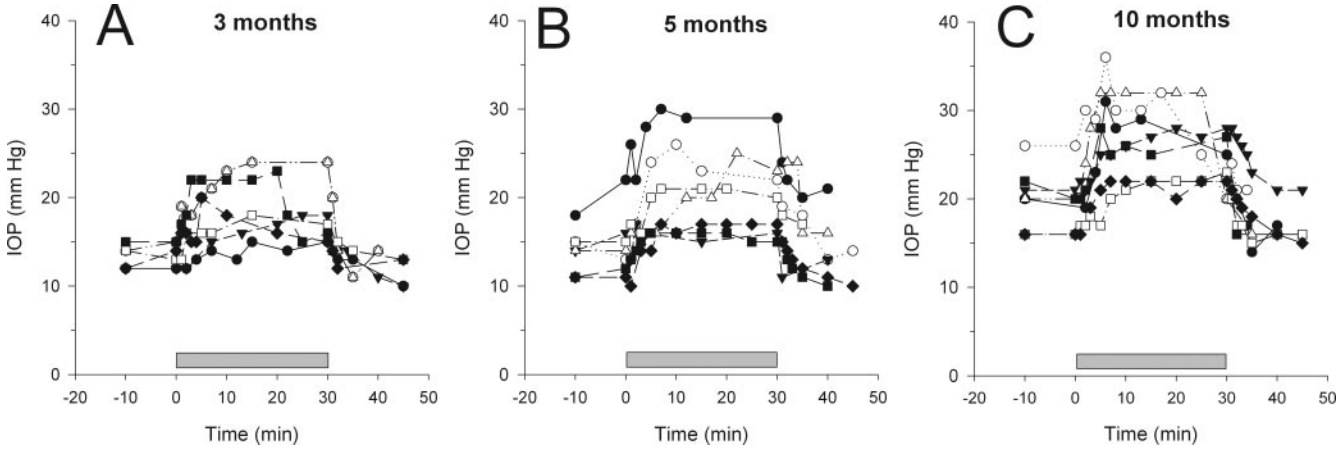

FIGURE 2. Time course of IOP changes during head-down (60°) body tilting (*gray bar* over the *x*-axis) in DBA/2J mice of different ages (**A**, 3 months; **B**, 5 months; **C**, 10 months). Different symbols correspond to individual mice.

Experiment 2: IOP Lowering

With the animal resting in a horizontal position, two baseline IOPs were measured. One intraperitoneal injection of mannitol 25% (2.5 g/kg; Hospira, Inc., Lake Forest, IL) was then administered, and IOP was measured approximately every 5 minutes over 1 hour. Mice were then allowed to recover from anesthesia and returned to their cages. One week later, IOP was again measured with the animal resting in a horizontal position as a control to show that IOP had recovered initial baseline values. Mice were then inserted in the stereotaxic holder for PERG recording in a horizontal position. After baseline PERG and FERG were recorded, one intraperitoneal injection of mannitol 25% was administered and PERG and FERG were recorded 20 to 60 minutes later. No further IOP measures were taken.

Time-Course of IOP Changes

In most eyes of each age group, the number of data points was sufficient to produce a mathematical fit of the time-course of IOP increase and decay. Data were well fitted with exponential rise and decay functions. The exponential increase is calculated as $IOP_{(t)} = {}_pIOP + {}_dIOP \cdot (1 - e^{-x/\tau})$; exponential decay is calculated as $IOP_{(t)} = {}_pIOP + {}_dIOP \cdot (e^{-x/\tau})$, where ${}_pIOP$ represents the plateau IOP, ${}_dIOP$ represents the delta IOP, and τ represents the time constant of IOP change. The time constant is the time required to change the baseline value by a factor of 1/e (63%).

RESULTS

Experiment 1: Effect of IOP Increase

Figure 2 displays the time-course of IOP changes measured in the left eyes of 21 mice of different ages under baseline condition (horizontal), tilted (60° head-down), and recovery (horizontal). On head-down positioning, the IOP progressively increased in all eyes and reached a relatively stable value. On horizontal repositioning, the IOP progressively returned to baseline values. The pupil size did not appreciably change during the entire observation period. The time courses of IOP rise and decay were symmetric, with a time constant on the order of 4 minutes, on average (IOP increase $\tau = 3.6 \pm 2.2$; IOP decay $\tau = 4.6 \pm 3.3$).

Average PERG amplitudes recorded with the mice in a horizontal position (baseline), 10 to 30 minutes in the head-down position (tilted), and 10 to 30 minutes after resuming the horizontal position (recovery) are displayed together with corresponding IOP and FERG amplitude (Fig. 3). Data were plotted on log scales, to normalize differences with respect to baseline conditions as well as relative changes of PERG amplitude with respect to IOP changes. The mean baseline IOP increased with increasing age (3 months, 13.2 ± 1.1 mm Hg; 5 months, 14.6 ± 3.2 mm Hg; and 10 months, 20.0 ± 3.4 mm Hg; ANOVA, $P < 0.01$). The increase in baseline IOP with age

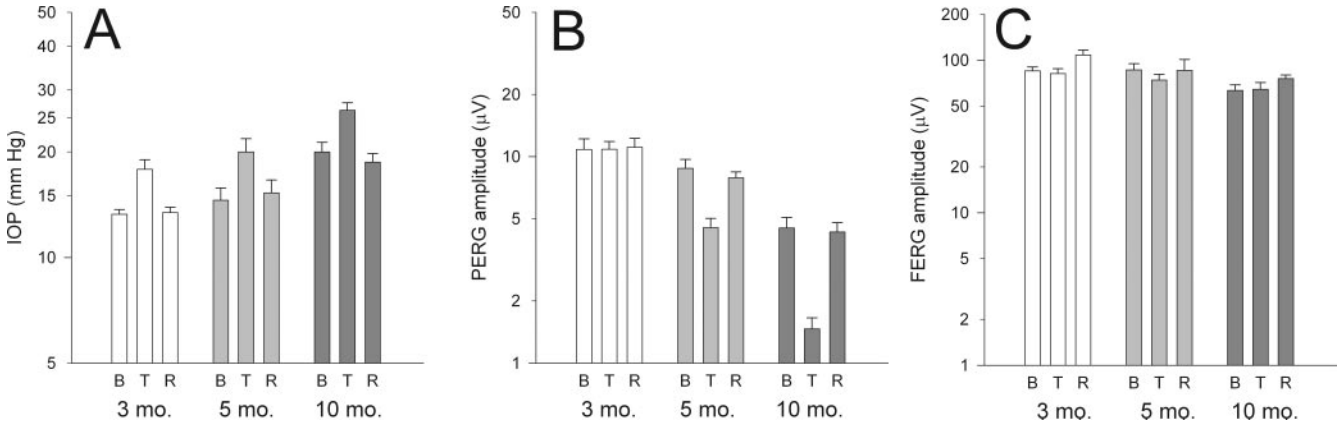

FIGURE 3. Average (±SEM) IOP (**A**), PERG amplitude (**B**), and FERG amplitude (**C**) in DBA/2J mice of different ages measured in a horizontal baseline condition (**B**), during head-down body tilting (T) and during recovery (R) in a horizontal position. Data are plotted on log scales to normalize relative changes.

was significant between the 10- and 3-month-old mice ($P < 0.001$) but not between the 5- and 3-month-old mice ($P = 0.33$). The ΔIOP (tilted minus horizontal baseline) was on the order of 5 mm Hg, and the relative change (tilted/horizontal baseline × 100) did not vary significantly with age (3 months, 33.8% ± 13.9%; 5 months, 37.8% ± 17.9%; and 10 months, 32.4% ± 10.7%, ANOVA, $P = 0.77$). The mean baseline PERG amplitude decreased with increasing age (3 months, 10.8 ± 3.5 μV; 5 months, 8.7 ± 2.4 μV; and 10 months, 4.5 ± 1.5 μV, ANOVA, $P < 0.01$). The decrease in baseline PERG amplitude with age was significant between the 10- and 3 month-old mice ($P < 0.001$) but not between the 5- and 3-month-old mice ($P = 0.28$). Different from IOP, the percentage of PERG amplitude change (tilted/horizontal baseline × 100) was strongly dependent on age (3 months, 3.4% ± 16.5%; 5 months, −46.8% ± 15.4%; and 10 months, −65.4% ± 14.0%; ANOVA, $P < 0.001$). In 3-month-old mice the PERG amplitude change was not significantly different from 0, whereas in the 5- and 10-month-old mice, PERG amplitude reductions in head-down position were highly significant ($P < 0.001$). Relative PERG amplitude reductions in the 10-month-old mice were significantly ($P < 0.05$) larger than those of 5-month-old mice. Note, by comparing Figure 3A with 3B, the age-dependent disproportion between logarithmic differences of IOP and PERG amplitude. In all mice, the PERG amplitude fully recovered baseline level when the mice were tested again in horizontal position. The head-down position did not cause significant changes in FERG amplitude. The peak latency of both PERG and FERG did not change significantly across conditions (not shown in the figures).

Mean PERG amplitudes recorded in baseline and tilted positions have been plotted against corresponding mean IOP values and the data fitted with linear regressions (Fig. 4). The correlation between PERG amplitude and IOP increased with increasing age of mice (3 months old: $r^2 = 0.04$, $P = 0.53$; 5 months old: $r^2 = 0.17$, $P = 0.14$; 10 months old: $r^2 = 0.58$, $P < 0.001$).

Experiment 2: Effect of IOP Decrease

Figure 5A displays the time course of IOP changes measured in eight mice (16 eyes) aged 11 months in horizontal position after intraperitoneal injection of 25% mannitol. The time course of IOP decay had a time constant on the order of 5 to 6 minutes (average $\tau = 5.5 \pm 3.5$). The pupil size did not appreciably change during the entire observation period. As shown in Figure 5B the untreated IOP was 26.3 ± 4 mm Hg, on average. Mannitol treatment consistently reduced the IOP to a relatively stable average of 16.3 ± 2.8 mm Hg (−38%; $P < 0.001$) over the next 20 to 60 minutes. When the same mice were tested 1 week later, IOP had recovered to baseline (mean 26.0 ± 4.3). PERGs and FERGs were then recorded before and 20 to 60 minutes after mannitol treatment. The baseline PERG amplitude of these 11-month-old DBA/2J mice (Fig. 5C) was typically very low (Saleh M et al., *IOVS* 2007;48:ARVO E-Abstract 210) and close to the noise level, as measured by recording a response to a pattern stimulus of 0 contrast.[11] After mannitol treatment, the PERG amplitude improved by approximately 83% ($P < 0.01$; Fig. 5C). Mannitol treatment had no significant effect on FERG amplitude (Fig. 5E), PERG latency, and FERG latency (data not shown).

Mean PERG amplitudes recorded before and after mannitol were plotted against corresponding mean IOP (Fig. 5E) and the data fitted by linear regression ($r^2 = 0.41$, $P < 0.001$).

Discussion

In agreement with previous results,[13] this study shows that it is possible to induce transient and consistent elevations of IOP in ketamine-xylazine anesthetized DBA/2J mice of different age by placing the animals in a 60° head-down position. The amount of IOP increase (mean, ~5 mm Hg) was larger than that (mean, 2.6 mm Hg) reported in ketamine-xylazine anesthetized NIH Swiss white mice in a 60° head-down position. The reason for this difference may be the different technique of IOP measurement. The present data were obtained with a noninvasive rebound tonometer (Tonolab) whereas previous results[13] were obtained by cannulating the anterior chamber (AC) with a water-filled microneedle connected to a pressure transducer.[24] However, baseline IOPs measured in this study (13.2 ± 1.1 mm Hg in 3-month-old mice) are similar to those measured with AC cannulation in 3-month-old NIH Swiss white mice (14.8 ±2.3 mm Hg,[25]16.5 ± 0.6 mm Hg[13]). In addition, IOP readings obtained with a rebound tonometer compare very well with IOP readings obtained with AC cannulation.[18,26] Overall, it appears more likely that the differences in posture-induced IOP elevation are due to genetic differences between DBA/2J and NIH Swiss white mice rather than the different techniques for measuring IOP.

Posture-induced IOP elevation is closely related to the increase of the episcleral venous pressure (EVP) as experimen-

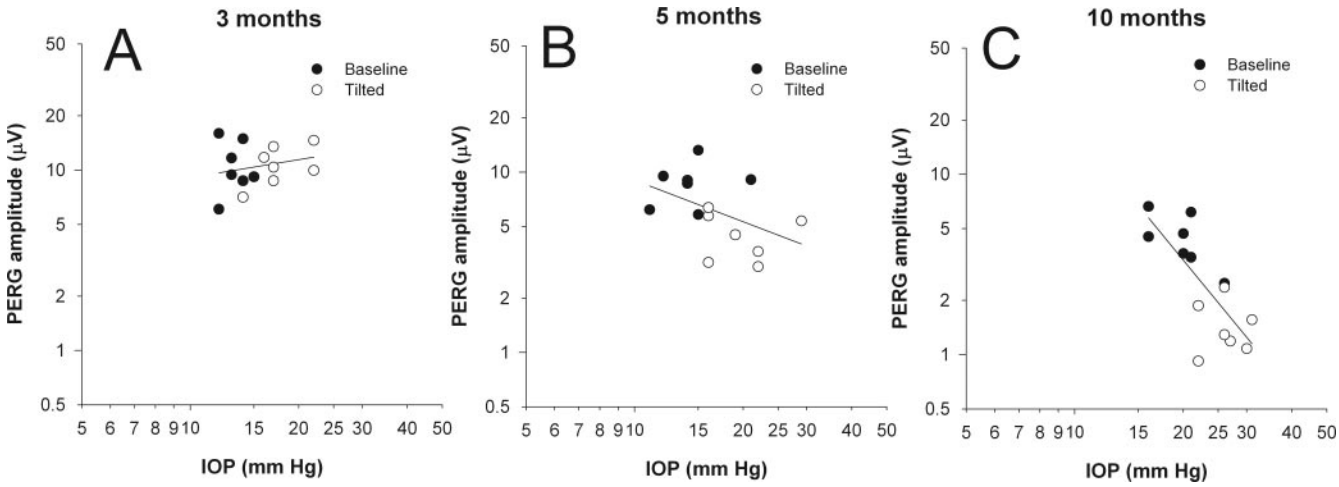

Figure 4. Changes in PERG amplitude and IOP after 60° head-down body tilting in DBA/2J mice of different ages (**A**, 3 months; **B**, 5 months; **C**, 10 months). Data have been fitted with linear regression lines.

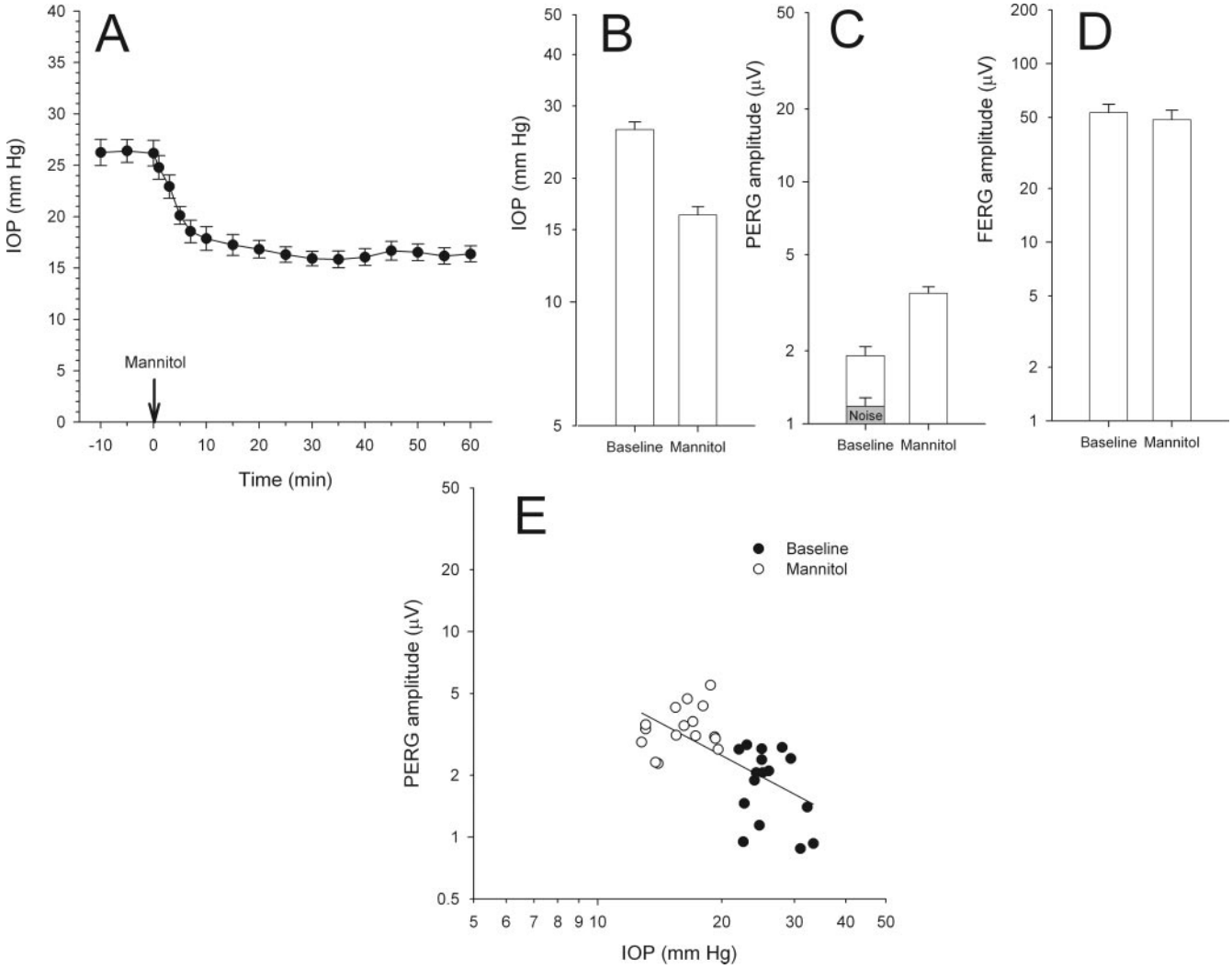

FIGURE 5. Effect of mannitol treatment on IOP and PERG in 11 month-old DBA/2J mice. (**A**) Time-course of IOP after mannitol treatment. (**B–D**) Mean (±SEM) IOP (**B**), PERG amplitude (**C**), and FERG amplitude (**D**). The *gray bar* in (**C**) represents the average (±SEM) noise level. Data are plotted on log scales to normalize relative changes. (**E**) Changes in PERG amplitude and IOP after mannitol treatment. Data have been fitted with linear regression lines.

tally demonstrated in humans[27,28] as well as mice.[13] The EVP increase in human subjects and mice is most likely caused by an increase in the volume of the choroidal vascular bed.[13,28,29] The time course of choroidal engorgement after head-down position resulting in elevated EVP is thought to be relatively brief (seconds). Our data show that the time course of IOP elevation is much longer (minutes). The gradual increase in IOP probably reflects the dynamic interaction between the rate of aqueous humor formation and the establishment of a new IOP equilibrium, to compensate for the increase in afterload to aqueous outflow (discussed in Refs. 30,31) A similar reasoning applies to the slow decay of IOP on horizontal repositioning. The mouse anterior chamber[32] and the turnover of aqueous humor[33] are comparable to those of humans. In human subjects, postural changes also induce changes in systemic blood pressure (e.g., Ref. 34) and ophthalmic artery pressure that may have a role in IOP regulation. These measures are not available for the mouse. However, Savinova et al.[35] did not find a positive correlation between blood pressure and IOP in different mouse strains in which blood pressure differed up to 36 mm Hg.

This study shows that postural IOP elevation induced reversible, age-dependent reductions of PERG amplitude in DBA/2J mice. The baseline IOP, the amount of IOP elevation on tilting, and the baseline PERG amplitude were similar in the 3- and 5-month-old mice. However, clear PERG amplitude reduction occurred in the 5-month-old mice but not in the 3-month-old mice. This indicates increased susceptibility of retinal neurons to IOP insult in the 5-month-old mice compared with the 3-month-olds. In the 10-month-old mice, the baseline IOP was increased, and the PERG amplitude decreased, compared with the 3-month-olds. IOP elevation on tilting exacerbated the preexisting PERG amplitude deficit to a larger extent than in the 5-month-old mice. In mice of all ages, posture-induced IOP elevation did not cause FERG changes, thereby excluding a generalized retinal effect and indicating that PERG changes reflect inner retina dysfunction.

This study also shows that intraperitoneally administered mannitol consistently reduced IOP by 38%, on average. Both the amount of IOP reduction and the time course of IOP changes are in good agreement with previous results.[15] In the 11-month-old DBA/2J mice, in which the baseline PERG was close to the noise level, IOP reduction resulted in a marked improvement of PERG amplitude (by ~83%, on average). Altogether, the findings indicate that a reduced PERG signal in the 11-month-old DBA/2J mice resulted, at least in part, from

the reduced responsiveness of viable retinal neurons rather than from lack of signaling in dead neurons. Reduced responsiveness was alleviated by IOP reduction. IOP reduction did not cause FERG changes, thus indicating that PERG changes reflected selective improvement of inner retina dysfunction.

Thus, in older DBA/2J mice RGC dysfunction appears to be strongly IOP dependent, since it was either exacerbated or alleviated, respectively, by experimental elevation or lowering of the IOP. This observation does not imply that IOP is the only causal factor in RGC dysfunction. In addition to elevated IOP, different insults may combine to induce RGC death in glaucoma.[36,37] In DBA/2J mice, these include excitotoxicity, axonal injury, glial activation, ischemia, and autoimmunity.[38–41] The age-dependent, IOP-independent increase in RGC susceptibility in mice has also been reported in response to optic nerve crush.[42] Finally, RGC susceptibility to optic nerve crush in mice depends on the genetic background.[43] Altogether, these results indicate that IOP initiates or amplifies pathogenetic processes of RGCs, resulting in progressive reduction of electrical responsiveness. Reduction of RGC electrical responsiveness is at least in part reversible by lowering IOP, thus indicating that a stage of IOP-dependent, reversible RGC dysfunction may precede cell death. The present findings support previous reports of PERG improvement after IOP reduction in patients with glaucoma and ocular hypertension (reviewed in Ref. 44).

Evaluation of PERG changes in response to artificial IOP modulation may represent a powerful tool for noninvasive evaluation of RGC susceptibility to IOP insult in genetically distinct mouse models of glaucoma.

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